

**EXPRESSION OF GENES ENCODING THIONINS AND LIPID-TRANSFER
PROTEINS. A COMBINATORIAL MODEL FOR THE RESPONSES OF
DEFENSE GENES TO PATHOGENS.**

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INTRODUCTION

Seeds normally accumulate high levels of proteins that are either toxic or inhibitory towards heterologous systems, including pathogens, pests and predators. This is the case of cereal kernels, where a substantial fraction of the non-storage proteins is represented by different families of these proteins (for reviews, see García-Olmedo et al.1989, 1992). The same types of proteins are often present in other tissues, either under normal development or under stress, including infection by pathogens. The thionins (THs) and the so-called non-specific lipid transfer proteins (LTPs) are two such families. Thionins were first reported in wheat endosperm (Balls et al., 1942) and their *in vitro* antipathogenic properties have been known for over 20 years (Fernandez de Caleyá et al. 1972), while cereal LTPs were first reported in barley aleurone (Mundy and Rogers 1986) and we have recently shown that different members of this family can be isolated from other barley tissues and that they are potent growth inhibitors of bacterial and fungal plant pathogens (Molina and García-Olmedo, 1991; Molina et al.1993; Molina and García-Olmedo, submitted). In this report, we will update our knowledge of the molecular biology and inhibitory properties of barley THs and LTPs, and will describe the developmental and pathogen-induced expression of the different genes encoding these proteins. Also, a working model for the response of defense genes against infection by different pathogens will be proposed.

STRUCTURAL TYPES AND THEIR GENETIC CONTROL

Thionins

Thionins are cysteine-rich polypeptides of about 5 kDa that have been found in a variety of tissues from different taxa (see García-Olmedo et al., 1989; 1992). Based on their similarity, known sequences can be grouped into at least 5 clusters or types (I-V), three of which (I,II,V) can be found in cereals such as wheat or barley.

The first type, which corresponds to the original purothionins from wheat endosperm (Balls et al. 1942), is 45 residues long, highly basic, with no acid residues, and has four disulfide bridges. There are one or two genes of type I per haploid genome in group-1 chromosomes of wheat and barley genomes. These genes encode precursor proteins in which the sequence of the mature protein is preceded by that of a signal peptide that is co-translationally processed and followed by that of a C-terminal, acidic peptide that undergoes a post-translational excision (Ponz et al. 1983; García-Olmedo et al. 1992).

Type-II thionins are less basic, with some acidic residues, and have an extra amino acid located in the central loop, as well as four disulfide bridges (Gausling 1987; Bohlmann and Apel 1987). Genes of this type, which seem to be present in 10-100 copies per haploid genome, have been located in chromosome 6H of barley and encode precursor proteins with the same characteristics as those of type I (Gausling 1987; Bohlmann and Apel 1987; Bohlmann et al.1988).

Type-V thionins are 37 residues long and have three disulfide bridges, two basic and two acidic residues, and a similar precursor structure as the previous types (Castagnaro et al. 1992). Genes of type V, one copy per haploid genome, have been located within a few kb of type-I genes in group-1 chromosomes in the wheat genomes (Castagnaro et al. 1992).

Lipid Transfer Proteins

Members of the LTP family have been characterized in barley

aleurone (Mundy and Rogers, 1986) and in green tissues, where we have recently reported a distinct subfamily of at least four members, which are structurally closer to LTPs isolated from other plant species than to the aleurone type (Molina et al.1993). Genes for these LTPs are located in chromosomes 3H (*Ltp2*, encoding protein Cw18; *Ltp4*, Cw21) and 7H (*Ltp3*, Cw20; Molina and García-Olmedo, submitted).

INHIBITORY PROPERTIES

Toxicity and Specificity

Both THs and LTPs are inhibitory and biocidal (at higher concentrations) towards bacterial and fungal pathogens. It has been shown that thionins are able to alter membrane permeability, indirectly inhibiting macromolecular biosynthesis (Carrasco et al. 1981), and to directly inhibit certain enzymes (Diaz et al. 1992 and unpublished), while the mechanism of action of LTPs remains unknown. As shown in Fig.1, different THs of types I and II show different activity towards a given pathogen, and the same is true for the barley LTP variants tested, but the relative ranking of the variants changes with the pathogen (i.e. *Clavibacter michiganensis* versus *Fusarium solani*).

The ability of THs to enhance resistance against certain pathogens *in vivo* has been recently reported (Carmona et al.1993).

Complementation and Synergism

Data in Fig.1 also show that while LTPs are about 20-fold more active than THs against the bacterial pathogen, they are somewhat less active than THs against the fungal one. Furthermore, we have found that the two types of proteins can act additively against the bacterium (not shown) and synergistically against the fungus (Fig.2). *Complementation*, as well as *additive* and *synergic* effects, might be relevant to explain interactions *in vivo*.

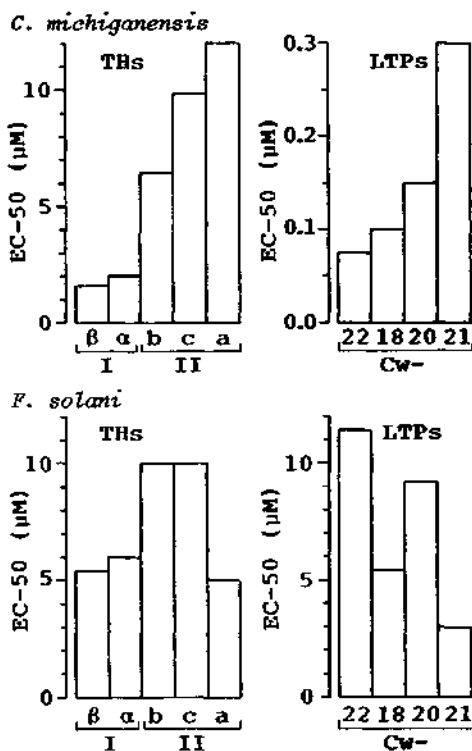


Fig.1. Growth inhibition of the bacterium *Clavibacter michiganensis* and the fungus *Fusarium solani* by TH and LTP variants from barley.

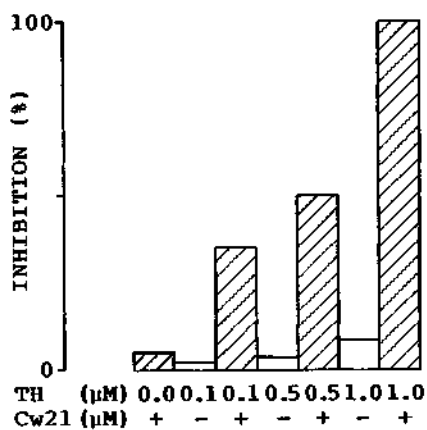


Fig 2. Synergism of THs (typeI) and LTPs (Cw21; 1μM) against *Fusarium solani*.

Resistance to Defense Proteins

Intraspecific variability of pathogen susceptibility to different defense proteins and the induction of resistance - including cross resistance - are being actively investigated by us, both to improve our understanding of compatible/incompatible interactions and because of the relevance of these properties when engineering plants for enhanced resistance. Examples of this intraspecific variability are shown in Fig.3.

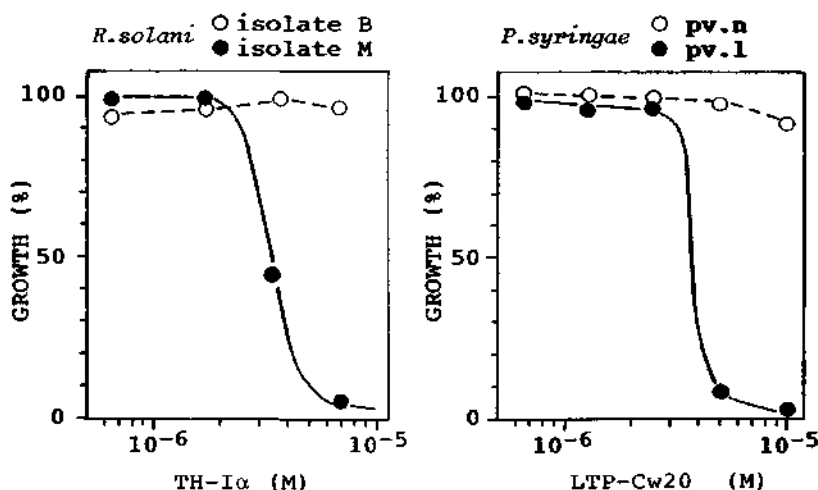


Fig.3. Differential *in vitro* resistance among pathogen strains.

EXPRESSION OF DEFENSE GENES

Normal Development

Expression patterns of three LTPs and of type-II THs are summarized in Table 1. All tissues tested, except endosperm, expressed the three LTPs investigated, and sequence-specific probes showed quantitative differences in the expression levels of the three genes. The probe used for the THs (type-II-specific) detected high levels of mRNA in etiolated leaves and lower levels in other tissues. Thionins of types I and V were restricted to endosperm (not shown). The LTPs described here are located in

vascular tissues and in the outer cell layer of the exposed surfaces of the plant, and can be washed out with buffer.

Table 1. Expression patterns* of *Ltp* and *Th* genes in barley

TISSUE	(age)	GENE			
		<i>Ltp2</i>	<i>Ltp3</i>	<i>Ltp4</i>	<i>Th</i>
Root	(young)	(+)	(+)	(+)	(+)
Leaf/dark	(young)	++	++	++	+++++
/light	(young)	++	++	+++	+
	(old)	+	+	(+)	(+)
Shoot apex		+	++	+	+++
Stem	(young)	+++	+++	+++++	+
	(old)	+	+	+	(+)
Spike/palea-lemma		(+)	++	+	(+)
/grain coats		(+)	+++	++	-
/rachis		(+)	++	+	(+)
/endosperm		-	-	-	-

* after overnight exposure; parenthesis indicate 5d exposure.

Responses to Abiotic Stimuli and to Pathogens

Young barley plants were exposed to different physical stresses and treated with different plant hormones and agrichemicals as summarized in Table 2. Steady-state mRNA levels of *Ltp* and *Th* genes were little affected by the physical treatments, except for moderate responses to salinity of two of the *Ltp* genes and the response to drought of *Th* genes. Absciscic acid increased the expression of both types of genes, and methyl jasmonate switched off *Ltp* genes, while markedly increasing the expression of *Th* genes. All other treatments had no detectable effects.

The current status of our analysis of the expression of these genes in response to pathogens is summarized in Table 3. Responses of the pathogenesis-related gene *Pr1* from barley (probe gift of W.Knogge) are also indicated.

Table 2. Responses (nX of basal levels) of *Ltp* and *Th* genes to abiotic stimuli (nd, not determined; -, no effect).

STIMULI	GENES			
	<i>Ltp2</i>	<i>Ltp3</i>	<i>Ltp4</i>	<i>Th</i>
Salinity	2X	-	2X	-
Cold	-	-	-	-
Drought	-	-	-	4X
Wounding	-	-	-	-
Methyl jasmonate	.05X	.06X	.12X	20X
Abscissic acid	5X	2X	3X	3X
Ethylene	-	-	-	-
Ethephon	-	-	-	nd
Salicylate	-	-	-	nd
Isonicotinic acid	-	-	-	nd

Table 3. Responses (nX of basal levels) of *Ltp* and *Th* genes to pathogens (-, no effect; C/I, compatible/incompatible).

PATHOGEN/CULTIVAR	C/I	GENES				
		<i>Ltp2</i>	<i>Ltp3</i>	<i>Ltp4</i>	<i>Th</i>	<i>Pr1</i>
<i>E.graminis</i> *						
(Av6)/Pallas	I	3X	3X	9X	3X	6X
(vir6)/Pallas	C	3X	3X	8X	3X	6X
<i>R.secalis</i> **						
/Atlas46	I	3X	-	10X	4X	10X
/Atlas	C	-	-	-	4X	-
/Turk	I	4X	-	16X	-	20X
/Hannchen	C	-	-	-	-	-
<i>P.syringae</i>						
(153)/Bomi	I	-	-	-	-	-
(DC3000)/Bomi	C	-	-	-	5X	4X

* filters from W.Knogge (Köln) and ** from L.Boyd (Norwich).

Different combinations of the genes investigated were affected in the different plant/pathogen interactions, and the extent and time-courses of the observed effects were also different. Thus, in the infection by *Erysiphe graminis*, both LTPs and THs were affected within the first few hours, before the progress of the compatible and the incompatible interactions can be distinguished microscopically from each other, according to Boyd (1993), whereas in the infection with *Rhynchosporium secalis*, LTPs were rapidly affected, while THs lagged behind by two days. Barley cv Turk, which failed to increase TH mRNA when challenged with *R.secalis*, was able to do so in response to some chemical treatments. In the case of the infection with *Pseudomonas syringae*, no hypersensitive reaction was detected in the incompatible interaction and none of the genes investigated was expressed above basal levels. This basal resistance was likely due to constitutive levels of defense proteins active against the pathogen.

A WORKING MODEL

The above observations suggest a number of features related to defense proteins that can be incorporated to current models of plant/pathogen interactions (Fig.4). These can be summarized as follows:

a) Different defense genes can be activated through totally or partially independent pathways and/or receptors, a combination of which are activated by a given pathogen in the same or in different sets of plant cells.

b) The elicited response might be gratuitous if the elicited proteins are not inhibitory (individually or in combination) against a particular pathogen, which is nevertheless seen by the plant.

c) A compatible interaction might result not only from failure of the plant to recognize a particular pathogenic strain, and to activate the appropriate defense gene(s), but also from the ability of the specific strain to resist inhibitor(s) to which other strains are susceptible.

d) Basal levels of defense proteins (for which additional roles in development are not excluded) are often above 1×10^{-5} moles/kg fresh weight, concentrations that should be sufficient to inhibit growth of many pathogens by a mechanism that does not imply a hypersensitive reaction.

e) The same defense proteins might be involved in all the different situations described above (b,c, and d).

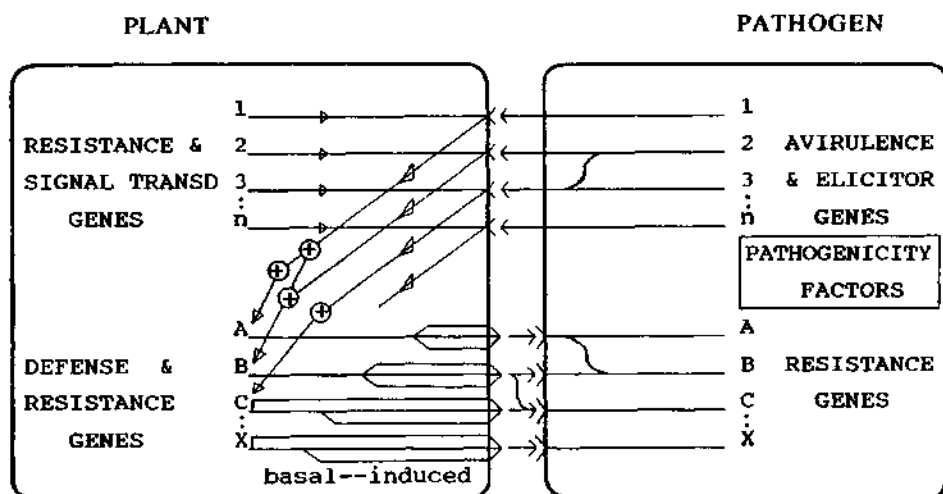


Fig.4. Schematic representation of activation of defense genes.

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REFERENCES

- Balls AK, Hale WS, Harris TH (1942) A crystalline protein obtained from a lipoprotein of wheat flour. *Cereal Chem* 19:279-288
- Bohlmann H, Apel K (1987) Isolation and characterization of cDNAs coding for leaf-specific thionins closely related to the

- endosperm-specific hordothionin of barley (*Hordeum vulgare* L.). *Mol Gen Genet* 207:446-454
- Bohlmann H, Clausen S, Behnke S, Giese H, Hiller C, Reiman-Philipp U, Schrader G, Barkholt V, Apel K (1988) Leaf-specific thionins of barley - a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defense mechanism of plants. *EMBO J* 7:1559-1565
- Boyd L (1993) Gene expression in barley in response to mildew infection. In "Engineering plants against pests and pathogens" Proc. Juan March Workshop, Madrid, Spain. p. 83.
- Carmona MJ, Molina A, Fernandez JA, Lopez-Fando JJ, García-Olmedo F (1993) Expression of the α -thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. *Plant J* 3:457-462
- Carrasco L, Vazquez D, Hernandez-Lucas C, Carbonero P, García-Olmedo F (1981) Thionins: plant peptides that modify membrane permeability in cultured mammalian cells. *Eur J Biochem* 116:185-189
- Castagnaro A, Marañón C, Carbonero P, García-Olmedo F (1992) Extreme divergence of a novel wheat thionin generated by a mutational burst specifically affecting the mature protein domain of the precursor. *J Mol Biol* 224:1003-1009
- Diaz I, Carmona MJ, García-Olmedo F (1992) Effects of thionins on β -glucuronidase in vitro and in plant protoplasts. *FEBS Lett* 296:279-282
- Fernandez de Caleyra R, Gonzalez-Pascual B, García-Olmedo F, Carbonero P (1972) Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro. *Appl Microbiol* 23:998-1000
- García-Olmedo F, Rodriguez-Palenzuela P, Hernandez-Lucas C, Ponz F, Marañón C, Carmona MJ, Lopez-Fando JJ, Fernandez JA, Carbonero P (1989) The thionins: a protein family that includes purothionins, viscotoxins and crambins. *Oxford Surveys Plant Mol Cell Biol* 6:31-60
- García-Olmedo F, Carmona MJ, Lopez-Fando JJ, Fernandez JA, Castagnaro A, Molina A, Hernandez-Lucas C, Carbonero P (1992) Characterization and analysis of thionin genes. In *Genes involved in plant defense* (Boller T, Meins F, eds) Plant Gene Research, pp 283-302. Springer-Verlag
- Gausing K (1987) Thionin genes specifically expressed in barley leaves. *Planta* 171:241-246
- Molina A, García-Olmedo (1991) Patent application P 9101258 (24.05.91) PCT/EP92/01130
- Molina A, Segura A, García-Olmedo F (1993) Lipid transfer proteins (nsLTPs) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. *FEBS Lett* 316:119-122
- Mundy J, Rogers, JC (1986) Selective expression of a probable α -amylase/protease inhibitor in barley aleurone cells: comparison to the barley α -amylase/subtilisin. *Planta* 169:51-63
- Ponz F, Paz-Ares J, Hernandez-Lucas C, Carbonero P, García-Olmedo F (1983) Synthesis and processing of thionin precursors in developing endosperm from barley (*Hordeum vulgare* L.). *EMBO J* 2:1035-1040